

# Anti-inflammatory Effect of *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. Rhizomal Extract and Its Phenolic Compounds in Lipopolysaccharide-Stimulated Macrophages

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## ABSTRACT

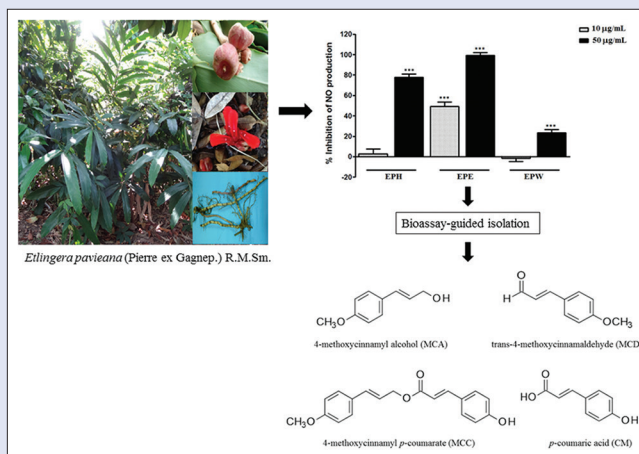
**Background:** In our continuing search for anti-inflammatory agents from Thai herbs, *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. showed potent inhibition on nitric oxide (NO) production in lipopolysaccharide (LPS)-induced macrophages. However, the mechanism behind its inhibitory effect has not been yet explored, and little is known regarding its bioactive compounds responsible for the anti-inflammatory effect. **Objective:** In the present study, anti-inflammatory effect of hexane, ethyl acetate, and water fractions of rhizomal ethanol extracts of *E. pavieana* was evaluated for their inhibition on NO production and mechanism in LPS-stimulated macrophages. Active compounds responsible for such anti-inflammatory activity were identified.

**Materials and Methods:** Inhibitory activities on NO production were performed in LPS-stimulated RAW264.7 macrophage. Cytotoxicity of plant extracts was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, mRNA and protein expressions by reverse transcription-polymerase chain reaction and Western blotting analysis, respectively. Anti-inflammatory compounds were isolated by activity-guided isolation technique using column chromatography. **Results:** Ethyl acetate fraction of *E. pavieana* (EPE) showed the most potent inhibitory effect on NO production in macrophages. EPE significantly decreased NO production and inhibited inducible nitric oxide synthase (iNOS) protein and mRNA expression in a dose-dependent manner. Furthermore, the level of nuclear factor-kappa B p65 subunit was markedly reduced in activated cells treated with EPE. Four phenolic compounds, 4-methoxycinnamyl alcohol (1), trans-4-methoxycinnamaldehyde (2), 4-methoxycinnamyl *p*-coumarate (3), and *p*-coumaric acid (4), were obtained from bioactivity-guided isolation technique. **Conclusions:** The anti-inflammatory property contained in *E. pavieana* rhizome extract and conferred through inhibition of iNOS expression, and NO formation provides scientific evidence and support for the development of new anti-inflammatory agents based on extracts from this plant.

**Key words:** 4-methoxycinnamyl *p*-coumarate, anti-inflammatory activity, *Etlingera pavieana*, inducible nitric oxide synthase, nitric oxide

## SUMMARY

- Ethyl acetate fraction (EPE) of *Etlingera pavieana* showed the most potent inhibitory effect on NO production in LPS-induced macrophages
- Four phenolic compounds, 4-methoxycinnamyl alcohol (1), trans-4-methoxycinnamaldehyde (2), 4-methoxycinnamyl *p*-coumarate (3) and *p*-coumaric acid (4), responsible for the anti-inflammatory effect of EPE were isolated.



**Abbreviations used:** EPE: Ethyl acetate fraction of *Etlingera pavieana*; EPH: Hexane fraction of *Etlingera pavieana*; EPW: Water fraction of *Etlingera pavieana*; NO: Nitric oxide (NO); LPS: Lipopolysaccharide; iNOS: Inducible nitric oxide synthase (iNOS); MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB: Nuclear factor-kappa B; DMSO: Dimethyl sulfoxide; EtOAc: Ethylacetate; MeOH: Methanol; AG: Aminoguanidine; DCM: Dichloromethane; MCA: 4-methoxycinnamyl alcohol; MCD: trans-4-methoxycinnamaldehyde; MCC: 4-methoxycinnamyl *p*-coumarate; CM: *p*-coumaric acid.

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## INTRODUCTION

Inflammation is a host defense mechanism against pathogenic challenges, to eradicate microbes or irritants associated with tissue injuries and to potentiate tissue repair.<sup>[1]</sup> Inflammation is traditionally defined by heat, redness, pain, and edema that reflect the actions of cytokines and other inflammatory mediators on local blood vessels.<sup>[2]</sup> Macrophages activated by pathogens and pro-inflammatory cytokines produce a series of mediators including nitric oxide (NO), prostaglandins, cytokines, and chemokines.<sup>[3,4]</sup>

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NO is a small free radical synthesized from L-arginine by NO synthase (NOS; EC 1.14.13.39)<sup>[5]</sup> and is important in numerous physiological processes.<sup>[6]</sup> Three forms of NOS have been identified: endothelial NOS, neuronal NOS, and inducible NOS (iNOS).<sup>[5]</sup> NO generated by iNOS plays a central role in inflammatory and immune reactions but when overproduced has been implicated in rheumatoid arthritis, septic shock, inflammatory bowel disease, cancer, neurodegenerative, and other disorders.<sup>[6]</sup> Expression of iNOS gene in lipopolysaccharide (LPS)-stimulated macrophages is mainly dependent on transcription factor nuclear factor-kappa B (NF- $\kappa$ B).<sup>[7]</sup> NF- $\kappa$ B is a hetero or homodimeric transcription factor composed of combinations of Rel family proteins. NF- $\kappa$ B is predominantly a heterodimer composed of p50 and p65 subunits in most cells. Activated NF- $\kappa$ B translocates to nucleus and binds to  $\kappa$ B sites of several pro-inflammatory genes including iNOS and promotes target gene expression.<sup>[6]</sup>

Plant taxa in family Zingiberaceae, widely distributed throughout the tropics, particularly Southeast Asia and in Thailand is represented by about 300 species belonging to 26 genera.<sup>[8]</sup> Rhizomes of one indigenous species, *Etingera pavieana* (Pierre ex Gagnep) R.M.Sm., are used to produce a spice and also for the treatment of digestive disorders, flatulence, and diuresis. All parts of the plant are aromatic.<sup>[9]</sup> Recently, some species of Zingiberaceae have been reported to have anti-inflammatory activity both *in vitro* and *in vivo*.<sup>[10-14]</sup> In our ongoing search for anti-inflammatory agents from Thai plants, *E. pavieana* rhizome extract was found to be a potent inhibitor of NO production in LPS-induced macrophages. However, the mechanism underlying its inhibitory effect remains unclear and little is known regarding its bioactive compounds. The present investigation examined the mechanistic anti-inflammatory action of *E. pavieana*, specifically its inhibitory activity on NO production in LPS-induced macrophages and identified the active compounds responsible for such anti-inflammatory activity.

## MATERIALS AND METHODS

### Chemicals

LPS (*Escherichia coli* serotype O111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and anti-mouse  $\beta$ -actin antibody were purchased from Sigma Chemical (St. Louis, MO, USA). Avian myeloblastosis virus reverse transcriptase and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG (H+L) were bought from Promega (Madison, WI, USA). Antibodies for p65 NF- $\kappa$ B and lamin A were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and for iNOS, from BD Bioscience (San Jose, CA, USA). Taq DNA polymerase was purchased from NEB (UK) and TRI reagent, from Molecular Research Center (Cincinnati, OH, USA).

### Plant material

*E. pavieana* (Pierre ex Gagnep.) R.M.Sm. (The Plant List record 244738) was collected from a local farm in Khung District, Chanthaburi Province, Thailand, in November 2011, and the species authenticated by Dr. B. Chewprecha, Department of Biology, Faculty of Science, Burapha University. A voucher specimen (KS-SCBUU-0012-1) was preserved at Faculty of Science, Burapha University.

### Plant extraction and isolation of bioactive compounds

Fresh rhizomes were washed with tap water, cut into thin slices, dried in a hot air oven at 50°C, and ground to a fine powder. Rhizome powder (1.2 kg) was extracted by ethanol (1:10) thrice at room temperature for 7 days and filtered under pressure through Whatman filter paper No. 3. Filtrate was pooled and evaporated by rotary evaporator. The ethanol extract (71 g) was then partitioned with hexane, ethyl acetate, and water, respectively. Then, solvents were filtered, removed *in vacuo*, and ultimately yielded

hexane fraction (EPH), ethyl acetate fraction (EPE), and water fraction (EPW) of *E. pavieana*, as 20.4 g (1.72%, w/w), 22.5 g (1.89%, w/w), and 27 g (2.27%, w/w), respectively. Dried powder of all fractions was dissolved in dimethyl sulfoxide and subsequently passed through a 0.22  $\mu$ M sterile filter before treating macrophage cells.

EPE fraction which exhibited the most NO inhibitory activity was subjected to silica gel flash column chromatography and eluted with a gradient of EtOAc/hexane (10%–80%) to yield five subfractions, SF1-SF5. Sub-fraction SF1 (1.17 g, 0.098% w/w) was recrystallized from MeOH/DCM as compound 1 (20.4 mg). Based on anti-inflammatory activity, subfraction SF1.1 was subjected to a silica gel column and eluted in gradients of increasing EtOAc (10%–30%) in hexane, to afford seven fractions (SF1.1.1-SF1.1.7). Subfraction F1.1.4 was chromatographed on silica gel using step gradients of DCM/hexane (40:60, 50:50, 60:40, and 70:30 v/v) to obtain six fractions (SF1.1.4.1-SF1.1.4.6). Subfraction F1.1.4.4 was chromatographed on silica gel using the same solvent system as that for subfraction F1.1.4 to give compound 2 (105 mg). Further, compound 2 (46.2 mg) was isolated from SF1.1.4.5 by sequentially using silica gel column chromatography eluted with 100% DCM.

Subfraction SF2 (1.72 g, 0.15% w/w) was, based on its NO inhibitory activity, further isolated by silica gel flash column chromatography using step gradients of MeOH:DCM (1:99%–5:95%, v/v) as an eluent to obtain compound 1 (91.8 mg) after recrystallization from MeOH/DCM. Subfraction SF2.1 was then separated by flash column chromatography using silica gel as the stationary phase with step gradients of MeOH/DCM (0:100, 1:99, 2:98, 3:97, 4:96, and 5:95 v/v) to obtain 7 sub-fractions (SF2.1.1-SF2.1.7). Sub-fractions SF2.1.2 and SF2.1.3 were recrystallized from MeOH/DCM as a yellow powder of compound 3 (141.8 and 118.8 mg, respectively). Then, sub-fractions SF2.1.2 and SF2.1.3 were mixed, subjected to silica gel column chromatography, and eluted with 100% DCM to produce compound 3 (250 mg).

Subfraction SF5 (1.75 mg, 0.15% w/w) was loaded onto a silica gel column and eluted with a gradient of MeOH/DCM (0:100, 1:99, 2:98, 3:97, 4:96, and 5:95 v/v) to obtain four sub-fractions (SF4.1-SF4.4). Subfraction SF4.3 was recrystallized from MeOH/DCM as a yellow solid of compound 4 (48.8 mg).

Proton NMR and Carbon NMR spectra were recorded on a Bruker AVANC 400 at 400 and 100 MHz, respectively. All spectra were measured in CDCl<sub>3</sub> solvent, and chemical shifts are reported as  $\delta$  values in parts per million relative to the solvent peak as an internal standard.

### Cell viability test by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Murine macrophage cell line RAW 264.7 was kindly provided by Prof. C. Kim, Laboratory of Leukocyte Signaling Research and Center for Advanced Medical Education by BK21 Project, Inha University, South Korea. Cells were suspended in Dulbecco's Modified Eagle Medium containing 10% heat-inactivated fetal bovine serum. Cell viability was evaluated from the ability of mitochondria to reduce tetrazolium salt MTT into MTT-formazan crystals.<sup>[15]</sup> Formation of formazan is proportional to the number of functional mitochondria in living cells.

### Nitrite measurement

Nitrite, a stable oxidation product of NO, was used as a measure of iNOS activity. Macrophages were co-treated with test compound and 1  $\mu$ g/mL LPS for 24 h. Nitrite present in the conditioned culture media was determined by spectrophotometric assay based on Griess reaction.<sup>[15]</sup>

### Reverse transcription-polymerase chain reaction

Isolation of total RNA was carried out using the TRI reagent according to manufacturer's instructions. Total RNA was reverse-transcribed to make

cDNA using AMV reverse transcriptase and oligo (dT)<sub>15</sub> primer. The reaction mixture of reverse transcription and polymerase chain reaction was performed as described by Srisook *et al.*<sup>[15]</sup>

## Western blot analysis

RAW 264.7 macrophages ( $1 \times 10^6$  cells) plated on 60 mm tissue culture plate were used for the preparation of proteins to be analyzed by Western blot assays. Cells were scraped in the presence of ice-cold lysis buffer containing 50 mM HEPES (pH 7.5), 2 mM EDTA, 50 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.3% Triton X-100, and a mixture of protease inhibitors (Complete mini, Roche, Germany). The cell lysate was sonicated twice for 1 min with 1 s interval with a Vibra-Cell ultrasonic processor set at 2W. Protein concentrations were quantified with a BCA protein assay kit. Equal amounts of cell protein were subjected to electrophoresis using 10% sodium dodecyl sulfate-polyacrylamide gels. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and nonspecific bindings were blocked with Tris-buffered saline-T buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% [v/v] Tween 20) containing 5% nonfat dried milk for 1 h at room temperature. The membrane was then incubated with primary antibodies of iNOS (1:2500 dilution) dissolved in 0.5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at 4°C overnight and  $\beta$ -actin (1:5000 dilution) dissolved in 5% BSA solution at room temperature. Subsequently, the membrane was incubated with goat anti-mouse or goat anti-rabbit IgG: HRP secondary antibodies for 1 h at room temperature. The specific protein bands on the PVDF membrane were visualized on X-ray film activated by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate. Intensities of each band signal were determined by densitometry using BIOPROFIL Bio-1D version 11.9 (Wilber Lourmat, France). Image densities of specific protein bands were normalized with the density of  $\beta$ -actin band as the internal control to compare amounts of specific protein accumulated in each sample.

Nuclear protein was the source for p65 NF- $\kappa$ B that was extracted as described by Srisook *et al.*<sup>[15]</sup> Protein concentration was determined with a Bradford protein assay kit. Levels of NF- $\kappa$ B p65 subunit in nuclear protein extract were determined by Western blotting analysis as described above. Proteins were transferred onto a PVDF membrane that was incubated first in a blocking solution and then overnight at 4°C in the presence of anti NF- $\kappa$ B p65 (1:500) and anti-lamin A (1:1000) antibody dissolved in 0.5% (w/v) BSA in PBS. Finally, the membrane

was transferred to a solution containing goat anti-rabbit IgG conjugated HRP secondary antibodies (1:5000 for NF- $\kappa$ B p65 and lamin A) for 1 h. The specific protein bands on the PVDF membrane were visualized on X-ray film activated by chemiluminescence. Image densities of specific bands for NF- $\kappa$ B p65 were normalized with the density of lamin A band.

## Statistical analysis

Results are means  $\pm$  standard deviation of at least three independent experiments. Statistical significance was tested using analysis of variance followed by Student's *t*-test for multiple comparison. Significance was accepted a  $P < 0.05$ .

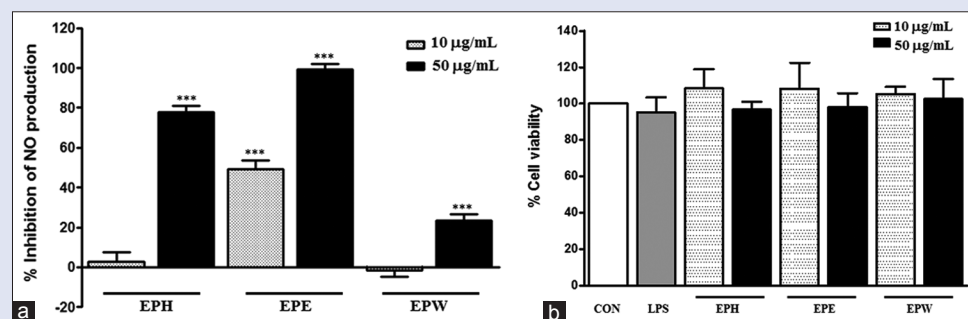
## RESULTS

### Effects of *Etilingera pavieana* extracts on nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophages

LPS caused a marked increase in nitrite concentration of media compared to unstimulated cells. The most potent inhibitory activity on NO production was found for the EPE compared to cells treated with LPS alone [Figure 1a]. Cell viability of all fractions was not significantly different from that of the control cells [ $P < 0.05$ ; Figure 1b]. EPE displayed a significant dose-dependent inhibitory effect on NO production with an IC<sub>50</sub> value of 16.28  $\mu$ g/mL [Figure 2a]. Furthermore, a number of viable activated cells were not significantly altered by EPE to 50  $\mu$ g/mL [Figure 2b].

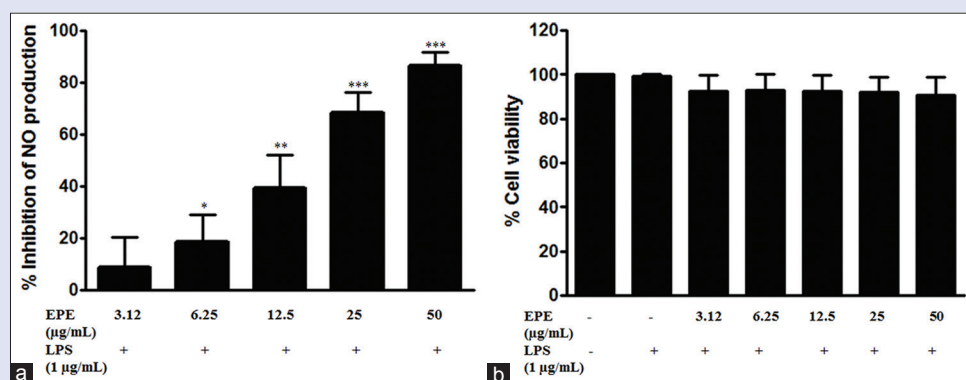
### Effect of ethyl acetate fraction of *E. pavieana* on inducible nitric oxide synthase expression and nuclear factor-kappa B p65 translocation in lipopolysaccharide-stimulated macrophage cells

iNOS protein was induced substantially in cells stimulated with LPS [Figure 3a]. Interestingly, EPE inhibited iNOS protein expression in a concentration-dependent manner compared to that by LPS [Figure 3a]. Moreover, synthesis of  $\beta$ -actin as a housekeeping protein was not affected significantly by LPS and EPE. We demonstrated also that iNOS mRNA level increased on exposure to LPS, and this increase was downregulated appreciably by EPE at 50  $\mu$ g/mL [Figure 3b]. The level of NF- $\kappa$ B p65 subunit increased in nuclear protein from cells treated with LPS, while EPE alone at 50  $\mu$ g/mL did not affect this level. Co-treatment with EPE and LPS markedly reduced NF- $\kappa$ B p65 subunit in a dose-dependent manner [Figure 4].

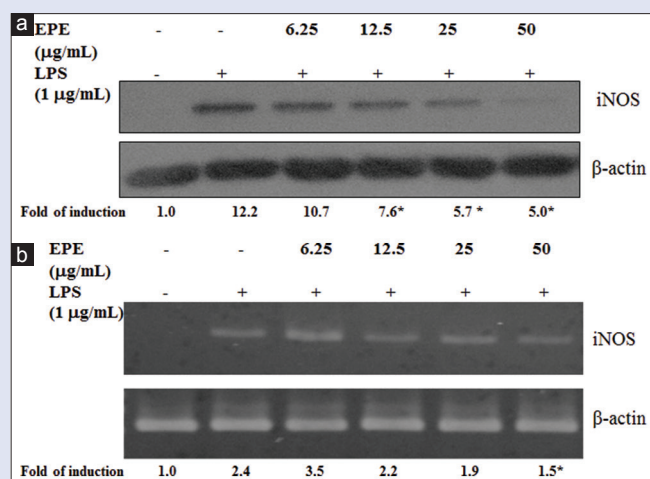


**Figure 1:** Effect of *Etilingera pavieana* fractions in lipopolysaccharide-stimulated RAW 264.7 macrophages. (a) Cells were co-incubated with 50  $\mu$ g/mL of each fraction of *Etilingera pavieana* and LPS (1  $\mu$ g/mL) for 24 h. Culture supernatants were collected subsequently and analyzed for nitrite production. Percentage inhibition of nitric oxide production from each treatment is given in relation to nitrite concentration of lipopolysaccharide-stimulated RAW264.7 macrophage cells. \*\*\* $P < 0.001$  versus lipopolysaccharide alone. (b) Viability of cells harvested 24 h after treatment with each fraction of *Etilingera pavieana* was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Each column shows the mean  $\pm$  standard deviation of three independent experiments with triplicate samples. CON: Unstimulated control cells, LPS: Lipopolysaccharide-stimulated cell, EPH: hexane fraction, EPE: Ethyl acetate fraction, EPW: Water fraction. (a: 730 mm  $\times$  840 mm; b: 741 mm  $\times$  421 mm)





**Figure 2:** Effect of ethyl acetate fraction of *E. pavieana* on nitric oxide production in lipopolysaccharide-stimulated macrophages RAW 264.7. (a) Cells were incubated with various concentrations of ethyl acetate fraction of *E. pavieana* in the presence of 1 µg/mL lipopolysaccharide for 24 h. Accumulated nitrite concentrations were determined in the medium. Percentage inhibition of nitric oxide production from each treatment is given in relation to nitrite concentration of lipopolysaccharide-stimulated RAW264.7 macrophage cells. (b) Cells were treated with various concentrations of ethyl acetate fraction of *E. pavieana* for 24 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each column shows the mean ± standard deviation of three independent experiments with triplicate samples. \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001 compared to the lipopolysaccharide-conditioned media (a: 695 mm × 491 mm; b: 716 mm × 505 mm)

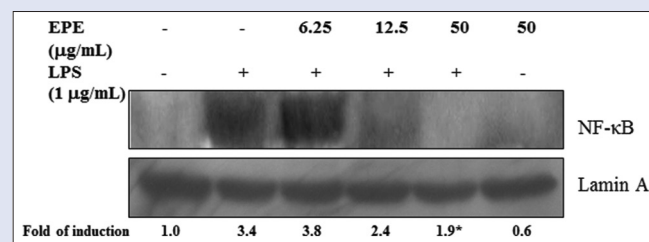


**Figure 3:** Effect of ethyl acetate fraction of *E. pavieana* on inducible nitric oxide synthase expression in lipopolysaccharide-stimulated macrophages. (a) Cells were incubated with various concentrations of ethyl acetate fraction of *E. pavieana* in the presence of 1 µg/mL lipopolysaccharide for 24 h. Cells were lysed, and inducible nitric oxide synthase as well as β-actin protein levels were determined by Western blot analysis. The immunoblot picture shown is a representative of three separate experiments. (b) Cells were incubated with various concentrations in the presence or absence of 1 µg/mL lipopolysaccharide for 12 h. Total RNA was extracted, and inducible nitric oxide synthase as well as β-actin mRNA levels were determined by reverse transcription-polymerase chain reaction. The gel picture shown is a representative of three separate experiments. \**P* < 0.05 compared to the lipopolysaccharide-treated cells (a: 690 mm × 234 mm; b: 707 mm × 281 mm)

### Isolation of bioactive compounds from ethyl acetate fraction of *E. pavieana*

In this study, isolation of bioactive compounds of EPE by NO inhibitory activity-guided fractionation technique yielded four phenolic compounds [Figure 5].

Compound 1 was isolated as a white solid: <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ3.47 (d, *J* = 7.4 Hz, 2H), δ3.80 (s, 3H), δ5.98–6.06 (m, 1H),

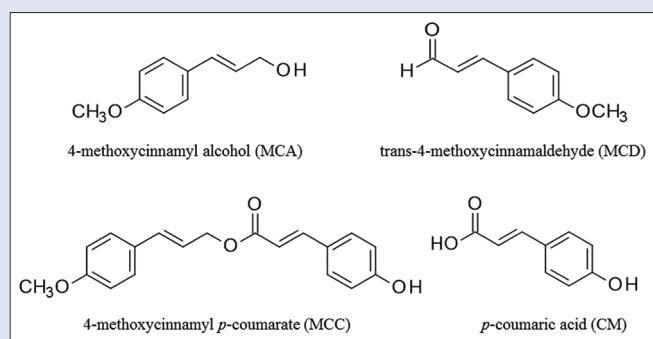


**Figure 4:** Effect of ethyl acetate fraction of *E. pavieana* on nuclear factor-kappa B p65 subunit nuclear translocation in lipopolysaccharide-stimulated macrophages. Cells were incubated with various concentrations of ethyl acetate fraction of *E. pavieana* and 1 µg/mL Lipopolysaccharide for 2 h. Nuclear protein extracts were prepared and analyzed by Western blot analysis. The gel picture shown is a representative of three separated experiments. \**P* < 0.05 compared to the Lipopolysaccharide-treated cells (684 mm × 254 mm)

δ6.39 (d, *J* = 15.5 Hz, 1H), δ6.83 (d, *J* = 8.3 Hz, 2H), δ7.27 (d, *J* = 8.3 Hz, 2H), <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz): δ42.7, 55.3, 114.0, 122.3, 127.6, 129.5, 133.1, 159.3. HRMS (ESI): C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>Na [M+Na]<sup>+</sup>, Anal. Cal. 187.0735. Compound 1 was identified as 4-methoxycinnamyl alcohol (MCA).

Compound 2 was isolated as a yellow solid: <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ3.81 (s, 3H), δ6.56 (dd, *J* = 7.8, 7.8 Hz), δ6.90 (d, *J* = 8.7 Hz, 2H), δ7.37 (d, *J* = 15.8 Hz, 1H), δ7.47 (d, *J* = 8.7 Hz, 2H), δ9.60 (d, *J* = 7.8 Hz, 1H), <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz): δ55.3, 114.4, 126.2, 126.6, 130.2, 152.6, 162.0, 193.6. HRMS (ESI): C<sub>10</sub>H<sub>10</sub>O<sub>2</sub>Na [M+Na]<sup>+</sup>, Anal. Cal. 185.0579, Found. 185.0567. Compound 2 was identified as 3-(4-ethoxyphenyl) prop-2-enal or trans-4-methoxycinnamaldehyde.

Compound 3 was isolated as a yellow solid: <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 400 MHz): δ3.81 (s, 3H), δ4.84 (d, *J* = 15.9 Hz, 2H), δ6.08 (s, 1H), δ6.19–6.26 (m, 1H), δ6.33 (d, *J* = 15.9 Hz, 1H), δ6.65 (d, *J* = 15.8 Hz, 1H), δ6.85–6.87 (m, 4H), δ7.38 (dd, *J* = 8.27, 8.15 Hz, 4H), δ7.67 (d, *J* = 15.9 Hz, 1H), <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100 MHz): δ55.3, 65.4, 113.9, 115.1, 115.9, 120.9, 127.0, 127.9, 128.9, 130.0, 134.0, 144.9, 157.9, 159.5, 167.5. HRMS (ESI): C<sub>19</sub>H<sub>18</sub>O<sub>4</sub>Na [M+Na]<sup>+</sup>, Anal. Cal. 333.1103, Found. 333.1102. Compound 3 was identified as 3-(4-methoxyphenyl)-allyl 3-(4-hydroxyphenyl)-acrylate or 4-methoxycinnamyl *p*-coumarate (MCC) from the spectroscopic data.<sup>[16]</sup>



**Figure 5:** Chemical structures of 4-methoxycinnamyl alcohol (1), trans-4-methoxy cinnamaldehyde (2), 4-methoxycinnamyl p-coumarate (3), and p-coumaric acid (4) (944 mm × 512 mm)

Compound 4 was isolated as a yellow solid: <sup>1</sup>H-NMR (MeOD, 400 MHz): δ6.28 (d, *J* = 15.8 Hz, 1H), δ6.81 (d, *J* = 7.9 Hz, 2H), δ7.45 (d, *J* = 7.9 Hz, 2H), δ7.59 (d, *J* = 15.8 Hz, 1H), <sup>13</sup>C-NMR (MeOD, 100 MHz): δ112.7, 113.9, 124.3, 128.1, 143.7, 158.2, 168.1. HRMS (ESI): C<sub>9</sub>H<sub>8</sub>O<sub>3</sub>Na [M + Na]<sup>+</sup>, Anal. Cal. 187.0371, Found. 187.0359. Compound 4 was identified as p-coumaric acid (CM).

NO inhibitory activity from EPE on phenolic compounds was greatest on compound 3, followed by compound 2 and compounds 1 and 4 [Table 1]. Interestingly, compound 3 exhibited a higher inhibitory effect on NO production than that of aminoguanidine, a known iNOS inhibitor<sup>[5]</sup> which had an IC<sub>50</sub> value of 50.3 ± 6.3 μM.

## DISCUSSION

Macrophages are the major source of iNOS-induced NO. Such NO production can be induced by inflammatory cytokines or bacterial products including LPS. The expression of iNOS and release of large amounts of NO are believed to play a significant role in the pathogenesis of various inflammatory and carcinogenic diseases.<sup>[6]</sup> In the present study, a LPS-induced inflammatory RAW 264.7 macrophage model was used to demonstrate inhibitory effects of NO production by *E. pavihana* extracts. Our previous study found that the organic solvent fractions of ethanol extract from *E. pavihana* rhizome at 50 μg/mL exhibited *in vitro* anti-inflammatory activity. In this study, EPE inhibited LPS-induced NO production in a concentration-dependent manner. Not all fractions of *E. pavihana* exhibited a cytotoxic effect on macrophages indicating that a reduction in NO production was not attributable to cell death [Figure 1b]. This is in agreement with previous reports that extracts from other species of Zingiberaceae also possess anti-inflammatory activity.<sup>[10-14]</sup>

EPE was chosen to determine the molecular mechanism of NO inhibition in LPS-treated RAW264.7 macrophages on the basis of its greater inhibitory effect than either hexane or water fractions. NO produced by iNOS is regulated mainly at the transcriptional level and at the translational level.<sup>[17]</sup> A reduction in protein synthesis results in a reduction of iNOS activity. iNOS protein was induced considerably in cells stimulated with LPS and suppressed by EPE. iNOS mRNA levels were also determined to examine whether the inhibitory effects of EPE on iNOS protein expression were related to the modulation of iNOS mRNA level. As shown in Figure 3, iNOS mRNA level was downregulated by EPE. Regulation of iNOS expression through the NF-κB pathway is an important mechanism in inflammatory processes and a potential site for intervention in inflammatory diseases.<sup>[18]</sup> Co-treatment with EPE and LPS markedly reduced NF-κB p65 subunit levels [Figure 4]. These data suggest that EPE could downregulate the expression of iNOS protein and mRNA, leading to a reduction of NO production in a sequential

**Table 1:** Nitric oxide inhibitory activity of bioactive compounds from ethyl acetate fraction of *E. pavihana*<sup>a</sup>

Compound	IC <sub>50</sub> (μM) <sup>b</sup>
Compound 1 (4-methoxycinnamyl alcohol)	>50
Compound 2 (trans-4-methoxycinnamaldehyde)	45.6±5.9
Compound 3 (4-methoxycinnamyl p-coumarate)	15.0±1.4
Compound 4 (p-coumaric acid)	>50

<sup>a</sup>All data are means±SD of at least three independent experiments with triplicate samples; <sup>b</sup>Under our experimental conditions. SD: Standard deviation

manner through the reduction of NF-κB p65 nuclear translocation. This is in accord with earlier observations that some Zingiberaceae species possess anti-inflammatory effects in macrophages due to the inhibition of the NF-κB signaling pathway.<sup>[12,18]</sup>

Earlier studies have isolated some chemicals from *E. pavihana* rhizomes;<sup>[19,20]</sup> however, until the present study, the chemical identity of extracts with anti-inflammatory properties has not been described. This study found the ethyl acetate fraction possessed the strongest inhibitory activity on NO production; therefore, it was further separated the active compounds by activity-guided isolation technique. Of the four compounds separated from this fraction, this is the first report on the isolation of compounds 1 and 4 from *E. pavihana* rhizomes. Compounds 2 and 3 were recently reported from *E. pavihana* rhizomes.<sup>[20]</sup> In addition to isolation from *E. pavihana*, compound 2 has been shown to be isolated from essential oil of *Illicium verum* Hook.<sup>[21]</sup> The compound possesses antihuman respiratory syncytial virus.<sup>[22]</sup> It was also shown antibacterial activity against *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Staphylococcus aureus*, and *Bacillus subtilis*.<sup>[23]</sup> However, this study showed that compound 2 suppresses NO secretion in LPS-induced macrophage with a moderate activity (IC<sub>50</sub> = 45.6 ± 5.9 μM).

Surprisingly, compound 3 was reported to be synthesized *in vitro* with a strong cytotoxicity to CNE and PC-3 cell lines.<sup>[16]</sup> In addition, it was weakly cytotoxic to KB, MCF7, and NCI-H187 cells.<sup>[20]</sup> In the present study, compound 3 showed high inhibitory activity (IC<sub>50</sub> = 15.0 ± 1.4 μM). It is interesting that compound 3 exhibited a more potent inhibitory effect on NO production than that by aminoguanidine (IC<sub>50</sub> = 50.3 ± 6.3 μM) under the same conditions.

Compound 1 was previously obtained from *Foeniculum vulgare* and exhibited cytotoxicity against MCF-7, HeLa, and DU145 cancer cell line.<sup>[24]</sup> Compounds 1 and 4 exhibited only weak inhibition of NO production (IC<sub>50</sub> >50 μM). This is in accord with a previous report that compound 4, isolated from corn bran, had only a weak NO inhibitory effect in LPS-treated macrophages.<sup>[25]</sup>

Based on the obtained data, we have tried to describe the relationship between the structure of these four phenolic compounds and their activity on NO inhibitory activity. Our study found compound 2, aldehyde derivative, to have more potent NO inhibitory activity than compound 1 with alcohol moiety, possibly a consequence of increased membrane permeability resulting from hydrophobicity of the aldehyde group. Interestingly, compound 3 MCC, an ester biosynthetically derived from compound 1 MCA and compound 4 (p-coumaric acid), showed stronger potent activity compared to its precursors. This result demonstrated that the ester moiety plays an important role in NO inhibitory activity. Previous work reported that the ester moiety can increase antioxidant activity of hydroxycinnamic acid resulting in higher inhibition of NO production due, at least in part, to its hydrophobicity.<sup>[26,27]</sup> Although the less polarity of ester group may increase NO inhibitory activity, the unique structure of compound 3, recently found in *E. pavihana*, may specifically affect NO inhibition. This is the first report of the inhibition of NO production by MCC (3).

## CONCLUSIONS

Collectively, our results suggest that chemicals in *E. pavieana* rhizome exert an anti-inflammatory effect in macrophages through suppression of NF- $\kappa$ B p65 nuclear translocation followed by a reduction in iNOS expression and NO production. Four phenolic compounds showing anti-inflammatory activity were isolated from *E. pavieana*. These findings provide scientific evidence to verify that *E. pavieana* rhizome might be a natural source for new anti-inflammatory agents.

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## Conflicts of interest

There are no conflicts of interest.

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